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PAPER

Electrochemical modification at multiwalled carbon nanotube electrodes with Azure A for FAD- glucose dehydrogenase wiring: structural optimization to enhance catalytic activity and stability

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Keywords: Azure A, diazonium electrochemical grafting, bioelectrocatalysis, FADGDH, mediated electron transfer, bioanode, multiwalled carbon nanotube

Abstract

Electrochemical grafting is a suitable technology for fabricating electrode surfaces with new chemical functionalities whilst maintaining the bulk properties of the electrode, and electrochemical amine oxidation and diazonium salt reduction are two widely used techniques to achieve this end. Herein, we report the electrochemical reductive grafting of Azure A onto multiwalled carbon nanotube (MWCNT) electrodes for the efficient wiring of flavin adenine dinucleotide (FAD) dependent glucose dehydrogenase. The diazonium salt of Azure A is formed *in situ* and subsequently grafted onto the electrode surface through electrochemical reduction. The formal potential of the resultant Azure-A-modified electrode shifted to −0.05 V vs. Ag/AgCl upon radical coupling to the MWCNT electrode. Electron transfer from FAD buried in the protein shell to the electrode via Azure A was then observed in the presence of glucose in the buffer solution. This study focused on the important effect of CNT mass loading on Azure-A loading as well as bioelectrocatalytic activity and storage stability. The three-dimensional porous structure of the MWCNT electrode was determined to be favorable for the immobilization of flavin adenine dinucleotide dependent glucose dehydrogenase and efficient electron transfer via the Azure-A functionalities. The optimized 300 μg CNT-loaded modified electrode on glassy carbon (3 mm diameter) retains its initial activity for 3 d and 25% of its initial activity after 10 d. Furthermore, we show that grafted Azure A is stably immobilized on the MWCNTs for 1 month; therefore, the limiting stability factor is enzyme leaching and/or deactivation.

1. Introduction

Tremendous effort has been directed toward the development of glucose electrodes that use enzymes as electrocatalysts to electrochemically oxidize glucose, with the electrode acting as the final electron acceptor. Glucose oxidase has been widely used as an electrocatalyst, but flavin adenine dinucleotide (FAD) dependent glucose dehydrogenase (FADGDH) has become the mainstream glucose oxidation electrocatalyst in recent years because FADGDH is not sensitive to oxygen. In contrast, glucose oxidase uses O_2 as a natural electron acceptor to generate hydrogen peroxide, which damages the enzyme electrode system [1–3]. In a fungal FADGDH, the FAD cofactor is surrounded by a protein shell, which typically requires a redox mediator to promote efficient electron transfer from FAD to the exterior electron acceptor. Osmium and ruthenium complexes, and organic mediators involving quinone and redox dyes, have been used as redox mediators [4–6]. For the application of FADGDH electrodes to enzymatic biofuel cells or for continuous-monitoring biosensors, the redox mediator needs to be immobilized on the electrode together with FADGDH [7, 8]. Organic mediators can be physically adsorbed onto carbon materials through hydrophobic interactions or π–π interactions, but are gradually desorbed from the electrode surface [9, 10]. Naphthoquinone derivatives have also been developed for FADGDH-mediator-modified electrodes [11, 12]; naphthoquinone is

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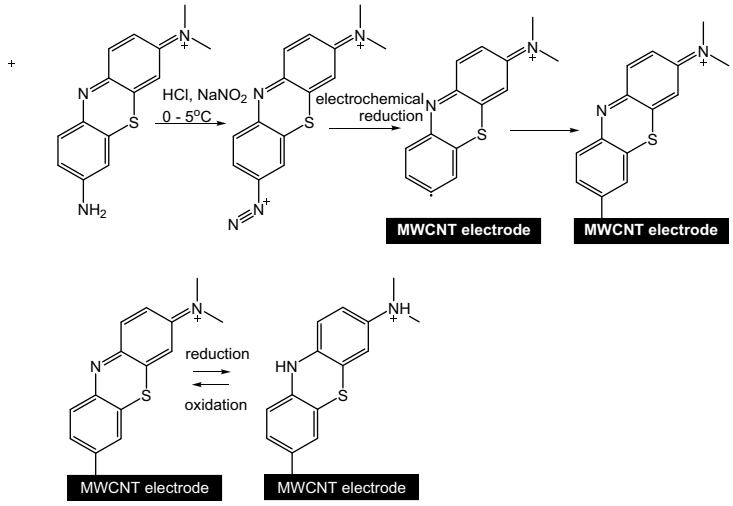

Scheme 1. Modifying an MWCNT electrode with Azure A by *in-situ* electrochemical grafting in aqueous acid.

immobilized by tethering to the backbone polymer or directly grafted onto FADGDH. Additionally, polymer-immobilized phenothiazine has been reported [13, 14]. Our research group previously reported that poly(methylene green), an electroanodically polymerized phenothiazine, acts as a redox mediator for FADGDH [15].

The electrochemical grafting of a redox mediator directly on an electrode surface is a desirable technology for fabricating the electrode surface [16–20], where amine oxidation and diazonium salt reduction are widely used techniques. Herein, we modified the electrode surface with Azure A using the *in-situ* diazonium-reduction technique. A variety of phenothiazines are available as redox mediators for FADGDH, but among these, Azure A contains one primary amino group, which readily facilitates control of the modification process (scheme 1). Azure A grafted on a screen-printed carbon electrode has been reported as an electrocatalyst for NADH oxidation [21]. As part of this study, we recently submitted an article that focuses on comparing adsorbed and covalently bound Azure-A-modified electrodes [22]. The present study further adds to our first studies on grafted Azure A as a redox mediator for the electron-transfer reaction between FADGDH and the electrode surface. In this work, we report new insight into the important effect of carbon nanotube loading and treatment on mediated bioelectrocatalytic glucose oxidation.

2. Experimental

2.1. Materials

FADGDH isolated from *Aspergillus terreus* and donated by Ikeda Tohka Industries Co., Ltd (2290 U mg^{-1}, Fukuyama, Japan). Azure A chloride (Sigma Aldrich, St. Louis, Missouri, USA; purity of 88% and a formal potential of −0.19 V vs. Ag/AgCl/KCl(sat.)) was used without further purification. The glucose stock solution was stored overnight to achieve mutarotative equilibrium. All experiments were conducted at room temperature (24 ± 1 °C). All reagents were of analytical grade.

2.2. Modifying glassy carbon (GC) electrodes with multiwalled carbon nanotube (MWCNTs)

First, 2.5 mg of MWCNTs (⩾95%, Ø = 9.5 nm, 1.5 μm, Nanocyl, Sambreville, Belgium) in 1.0 ml of 1-methyl-2-pyrrolidone (NMP) was ultrasonicated for 30 min. A 20 μl aliquot of the obtained homogeneous dispersion was applied to a GC electrode (Ø = 3.0 mm, 0.0707 cm^2, ALS, Tokyo, Japan) and dried at 60 °C for 24 h to afford an electrode referred to as 'MWCNT-E'; 50 μg of the MWCNTs were loaded unless otherwise stated. At higher MWCNT loadings, the MWCNT ink was applied to the electrode and dried, with the process repeated until the desired amount of MWCNT had been attained. Prior to electrochemical derivatization, the electrode was washed with methanol, followed by distilled water, unless otherwise stated.

2.3. Modifying MWCNT electrodes with Azure A

Azure A (14.5 mg) was dissolved in 5.0 ml of 0.5 M HCl solution (concentration of Azure A was 10 mM). After the solution was cooled to 2 °C, 6.9 mg of $NaNO_2$ dissolved in 5.0 ml of cold water (2 °C, 20 mM) was slowly added to the Azure A solution and stirred for 10 min to complete the formation of the diazonium salt. MWCNT-E was electrochemically modified in the Azure A diazonium solution using a Pt wire counter

electrode and a Ag/AgCl (saturated KCl) reference electrode. Cyclic voltammetry was performed in the 0 to −0.5 V potential range at a scan rate of 0.1 V s^{-1} for five cycles. All potentials are reported versus the Ag/AgCl (sat. KCl) reference electrode. After being electrochemically modified, the electrode surface was rinsed with distilled water, followed by methanol. The amount of Azure A on the modified electrode was determined based on the oxidation peak area of the voltammogram obtained in phosphate buffer solution (pH 7.0).

2.4. Enzyme modification and electrochemical oxidation of glucose catalyzed by FADGDH and mediated by Azure A on MWCNT electrodes

FADGDH was dissolved in phosphate buffer (0.1 M, pH 7.0) (the concentration of FADGDH was 1.0 mg ml^{-1}). The Azure-A-modified electrode was dipped in the FADGDH solution for 12 h at 4 °C. Prior to any electrochemical experiment, the electrode was washed with distilled water, followed by phosphate buffer solution, to remove the weakly adsorbed FADGDH.

The FADGDH-modified electrode, as the working electrode, was subjected to cyclic voltammetry and chronoamperometry. A Pt counter electrode was used with a Ag/AgCl reference electrode. The electrolyte solution consisted of 0.1 M phosphate buffer solution (pH 7.0) containing 0.1 M glucose, and the potential was scanned from −0.2 to 0.3 V and at 10 mV s^{-1} during cyclic voltammetry; a constant electrode potential of 0.2 V was applied during chronoamperometry studies.

3. Result and discussion

3.1. Modifying CNT electrodes with Azure A

Figure 1(A) shows cyclic voltammograms of the diazonium salt of Azure A in aqueous acidic media on the MWCNT-E. The first cycle (blue) of the cyclic voltammogram shows a single reduction peak in this potential range. During the second (red) and third (green) cycles, these reduction waves decrease in intensity and the cyclic voltammogram only exhibits a small reduction current, which suggests the presence of a grafted layer [16]. The cathodic peak starts at −0.2 V, which corresponds to the reduction of the aryldiazonium cations to aryl radicals that then bind covalently to the carbon surface [21]. The decline in the reduction current with increasing number of scans is consistent with the grafted layer restraining further electrochemical reduction of the diazonium salt, and consequently its own growth.

Figure 1(B) shows cyclic voltammograms recorded for Azure-A-modified electrodes washed with either water (blue) or water followed by methanol (red) in 0.1 M phosphate buffer solution (pH 7.0). The voltammogram of the modified electrode washed with only water shows two clear surface-confined redox reactions with apparent redox potentials of −0.02 V and −0.25 V. Notably, Azure A dissolved in the phosphate buffer solution (pH 7.0) at a pristine MWCNT-E electrode displays only one redox couple with a mid-point potential of −0.25 V (data not shown). In another control experiment, MWCNT-E was dipped in the Azure A diazonium solution without an applied potential. Only one redox couple was observed in phosphate buffer (pH 7.0) at −0.02 V (data not shown), suggestive of the spontaneous grafting of a polymer-type film of Azure A [23]. The redox couple at low potential for the Azure-A-modified MWCNT-E corresponds to the redox reaction of adsorbed monomer-type Azure A on the MWCNT-E. The small negative shift for adsorbed Azure A compared to dissolved Azure A indicates that adsorbed Azure A is more difficult to reduce, which appears to be consistent with the effective deposit of Azure A monomers.

The red curve in figure 1(B) is the voltammogram recorded for an Azure-A-modified electrode washed with water and then with methanol. The redox couple at low potential was dramatically less intense while the redox couple at high potential was not significantly different compared to the electrode prepared without methanol rinsing. Polymer-type Azure A covalently grafted on the electrode surface was found to be stable even after washing with methanol [24]. The disappearance of the lower potential redox couple with methanol rinsing is clear evidence for the removal of non-covalently adsorbed Azure A. The amount of electrografted polymer-type Azure A was determined to be 12.5 ± 0.5 nmol cm^{-2}, as calculated from the anodic peak area of the voltammogram obtained in phosphate buffer (figure 1(B)).

Figure 1(C) shows stability-testing data for the Azure-A-modified MWCNT-E in phosphate buffer (pH 7.0). The CVs are recorded after 0 (red), 7 (purple), 14 (blue), and 30 d (green). The electrode was stored in phosphate buffer solution (pH 7.0) in a refrigerator at 4 °C until use. The oxidation and reduction peak currents and potentials corresponding to polymer-type Azure A did not significantly change over 1 month. The grafted redox molecules on the MWCNT on the GC electrode are therefore very stable in phosphate buffer solution at neutral pH, as expected for covalently attached molecules.

Figure 2 shows the dependence of MWCNT loading on the electrochemical response, where panel (A) displays the cyclic voltammograms of an unmodified MWCNT-E in phosphate buffer (pH 7) at different MWCNT loadings (50, 100, 200, and 300 μg/electrode). Charging currents of 45, 90, 180, and 270 μA cm^{-2} were used for 50, 100, 200, and 300 μg MWCNT loadings, respectively. The charging current increases

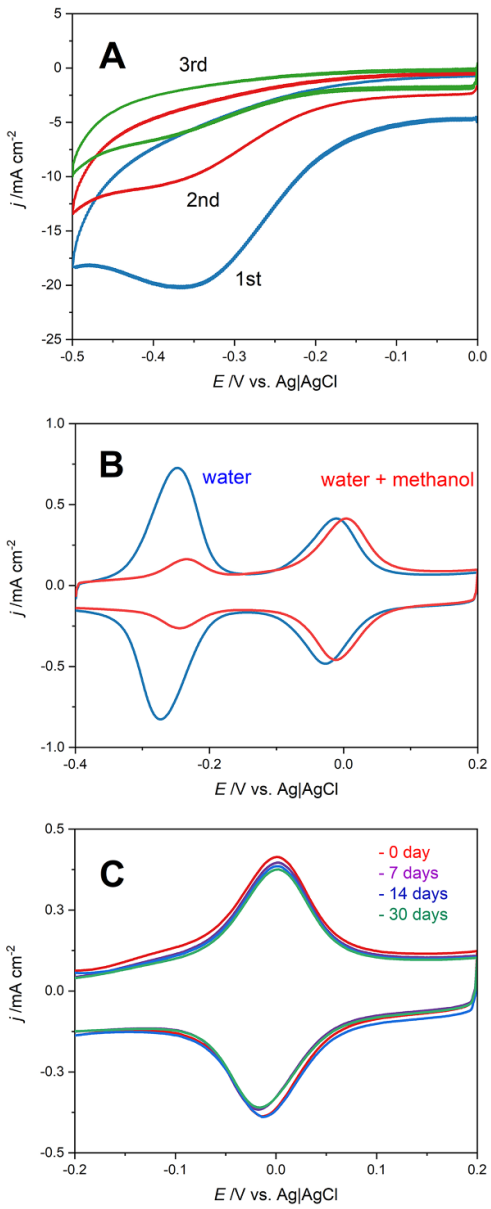

Figure 1. (A) Cyclic voltammograms for the reduction of *ca.* 5 mM azure A diazonium recorded at a scan rate of 0.1 V s^{-1} in 0.25 M HCl and 10 mM $NaNO_2$ as the supporting electrolyte; the first three cycles are shown: 1st (blue), 2nd (red) and 3rd (green). (B) Cyclic voltammograms of an Azure A-modified MWCNT electrode washed with water (blue), followed by methanol (red), in phosphate buffer (pH 7.0) at a scan rate of 10 mV s^{-1}. (C) Stability testing of an Azure-A-modified MWCNT electrode after washing with methanol by recording cyclic voltammograms in phosphate buffer (pH 7.0) at a scan rate of 10 mV s^{-1} after 0 (red), 7 (purple), 14 (blue), and 30 d (green). The electrodes were stored in a refrigerator in phosphate buffer (pH 7.0) at 4 °C until used.

proportionally with increasing MWCNT loading, which suggests that the electrochemically active surface area is proportional to the amount of MWCNTs in the studied range, consistent with good electrolyte accessibility in the three-dimensional (3D) nanostructured CNT film. The shape of the voltammogram also indicates that charge transfer through the MWCNT layer is very fast (= low internal resistance) over the potential window of interest for the bioelectrocatalytic oxidation of glucose. Fast charge transfer is related to a good ratio between oxygenated surface defects and the areas of the extended π-system of the outer wall of this carbon nanotube material [25]. The oxygen functionalities enable good wetting capacities with buffer solutions [26] while concurrently reducing the electrical inter-tube resistance [27]. The graphitized areas facilitate the stable assembly of the MWCNT deposits through π–π stacking interactions that prevent the collapse of the porous structure at higher loadings [28]. MWCNTs were modified with Azure A in the same manner as MWCNT-E (50 μg) and used in the experiments shown in figure 1. The effect of the MWCNT loading on the voltammogram that represents the electrochemical behavior of modified Azure A on the electrode is shown in figure 2(B); these voltammograms were also acquired in 0.1 M phosphate buffer (pH 7.0). The peak current increases linearly with increasing MWCNT loading on the electrode. The amounts of

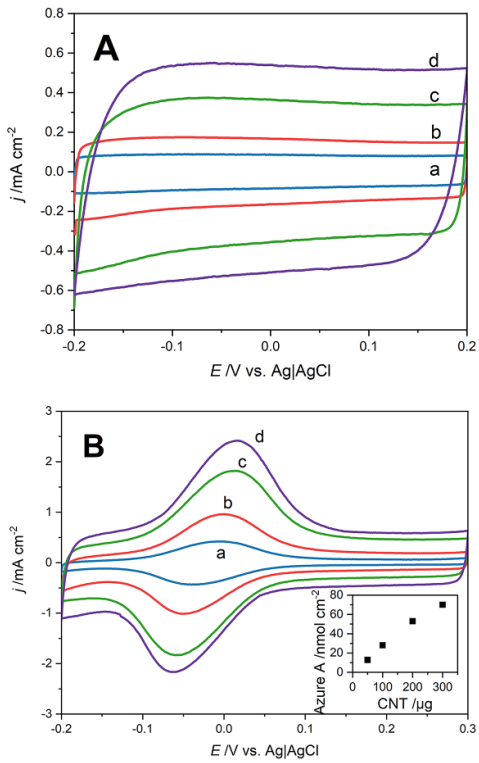

Figure 2. Cyclic voltammograms of (A) unmodified and (B) Azure-A-modified MWCNT electrodes in 0.1 M phosphate buffer (pH 7.0) at 10 mV s^{-1}. MWCNT loadings are 50 (blue curve a), 100 (red curve b), 200 (green curve c), and 300 (purple curve d) μg/electrode.

modified Azure A calculated from the peak current areas are 13, 28, 55, and 70 nmol cm^{-2}, which are proportional to the amounts of MWCNTs on the electrode surfaces. This result confirms that higher loadings of the Azure A mediator can be obtained by increasing the amount of MWCNTs, and furthermore, that electrolyte accessibility is not hindered by the increasing presence of Azure A functionalities.

3.2. Glucose oxidation catalyzed by FADGDH on Azure-A-modified MWCNT electrodes

Figure 3(A) shows voltammograms for the FADGDH-modified electrode in the presence (solid blue line) and absence (dotted blue line) of 100 mM glucose in phosphate buffer, which is much higher than the apparent Michaelis constant value of 25 mM and the limiting glucose concentration of 75 mM [22]. The anodic current in the presence of glucose starts to increase above the background current in the absence of glucose at −0.05 V, and the catalytic current gradually increases at a potential above 0.1 V; the reduction current is unaffected by the presence or absence glucose. The catalytic current increases with an increase in the amount of MWCNTs on the electrode but does not increase further, even if the MWCNT amount (and therefore the amount of Azure A) is increased beyond 200 μg. The catalytic current density at 0.3 V is 1.8, 3.2, and 5.6 mA cm^{-2} for MWCNT loadings of 50, 100, and 200 μg, respectively. The catalytic current increases proportionally with an increase in the MWCNT loading (i.e. electrode surface area). For a 300 μg MWCNT loading, the limiting step is the mass transfer of glucose from the bulk solution to the enzyme through the thick MWCNT matrix. An alternatively possibility is that the enzymes were not effectively adsorbed throughout the thicker MWCNT deposit, limiting the maximum optimal loading to 200 μg. It is possible that both effects operate together.

The stability of the modified electrode with immobilized FADGDH was also tested by performing periodic fixed-potential experiments for 5 min at 0.2 V, each time in fresh phosphate buffer solution (pH 7.0). The electrode was stored in a phosphate buffer solution (pH 7.0) in a refrigerator at 4 °C between experiments. The electrochemical glucose oxidation activity on the MWCNT (50 μg)-modified electrode decreased linearly with increasing storage time (days), with no glucose oxidation current finally observed after 7 d of storage. The redox peak currents of the voltammograms corresponding to Azure A obtained in the pH 7.0 buffer solution without glucose did not change during this period (data not shown), which clearly indicates that the decrease in stability is linked to the denaturation of FADGDH and/or its desorption from the MWCNT-E, rather than the decomposition or desorption of Azure A and/or CNTs on the electrode. Furthermore, this result highlights the good physical and electrochemical stability of the diazonium-derived

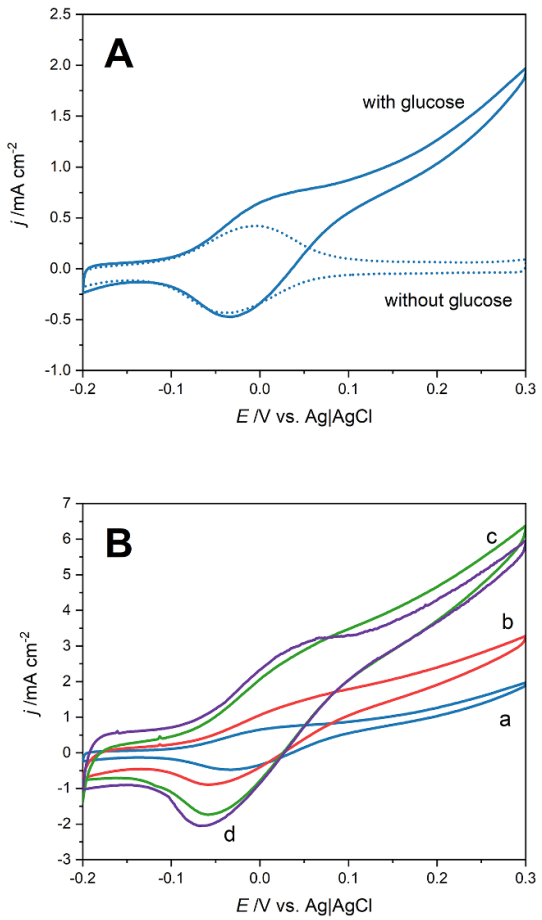

Figure 3. Cyclic voltammograms for the FADGDH-modified Azure A MWCNT-E (A) in the presence (solid line) and absence (dotted line) of 0.1 M glucose in phosphate buffer (pH 7.0) at 10 mV s^{-1}. The MWCNT loading is 50 μg/electrode. (B) Dependence of MWCNT loading: 50 (blue curve a), 100 (red curve b), 200 (green curve c), and 300 (purple curve d) μg/electrode in the presence of 0.1 M glucose in phosphate buffer (pH 7.0) at 10 mV s^{-1}.

modified electrode. FADGDH in a porous carbon electrode (MgO-templated mesoporous carbon) can retain its bioelectrocatalytic activity after storage in buffer solution at 5 °C over 7 months [8]. This may suggest that the main limitation of FAD-GDH is more related to enzyme desorption from the MWCNT matrix rather than deactivation, although in the cited work a hydrogel-polymer system likely enhances enzyme stability. We observed very limited stability of only a few days for the 50 μg MWCNT (figure 4), consistent with electrostatic interactions between the overall negatively charged enzyme (pI = 4.4) and positively charged Azure A grafted on the MWCNT surface not playing important roles. In contrast, the electrochemical glucose oxidation activity on the MWCNT (300 μg)-modified electrode showed significantly improved stability with increasing MWCNT loading, highlighting the more-important role of the porous CNT structure than the electrostatics of the Azure A layer [22]. The 300 μg MWCNT-E exhibited very good activity for the first few days with no significant loss in stability, compared to a stability loss of 40% after 3 d for the 50 μg MWCNT-E. The catalytic stability of the 300 μg MWCNT-E begins to decrease after 3 d of storage and retained almost 30% of its initial current density after 10 d.

4. Conclusion

Immobilization of a redox mediator and optimization of the 3D electrode structure are key technologies that increase the stability of a FADGDH-based electrode. Herein, Azure A is immobilized on MWCNTs through the formation of covalent bonds by the *in-situ* diazonium salt reduction technique. The grafted Azure A is very stably immobilized on the MWCNTs for 1 month in phosphate buffer at neutral pH and room temperature without loss of redox activity. Electron transfer from FAD buried in the protein shell to the electrode via Azure A is observed in the presence of a high concentration of glucose in the buffer solution. We reveal the importance of the CNT loading on the catalytic stability of the Azure-A–FAD-GDH electrode, showing that an increase in the CNT loading can help to maintain the initial high activity for 3 d while also providing around 30% of its initial activity after 10 d of storage in neutral buffer solution. The improvement

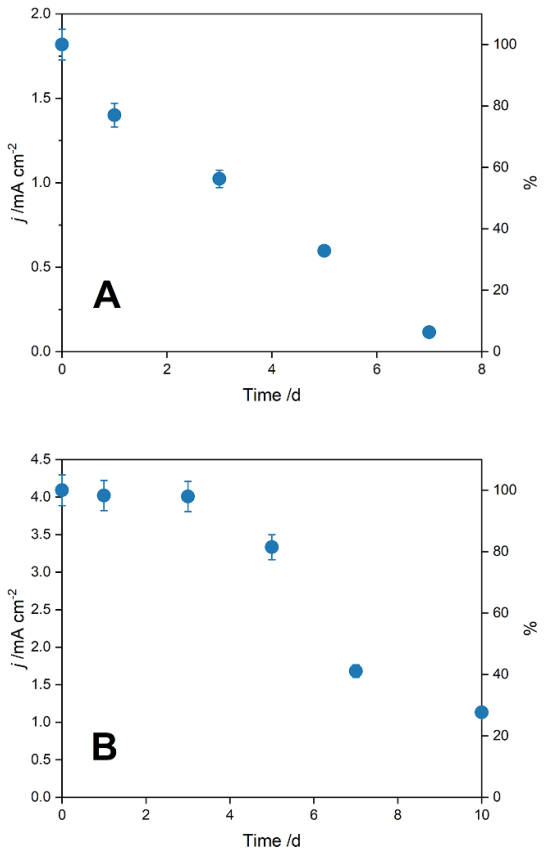

Figure 4. Storage stability of FADGDH–Azure-A-MWCNT-E. Glucose oxidation current recorded from amperograms at 0.2 V at room temperature in phosphate buffer (pH 7.0) containing 0.1 M glucose is plotted as a function of storage time. The MWCNT loadings are: (A) 50 μg and (B) 300 μg.

in stability highlights the importance of the 3D structure with a macro–meso porous hierarchical structure that is more favorable for the entrapment of FADGDH by the surrounding Azure-A-grafted MWCNT matrix, whilst also enabling efficient electrolyte diffusion and electron transfer. Further improvement is possible using enzyme crosslinkers such as poly(ethylene glycol) diglycidyl ether or glutaraldehyde and/or a hydrogel to enhance the fixation and stability of the enzyme at the electrode.

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